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High Performance Liquid Chromatography Method for Rapid and Accurate Determination of Homocysteine in Plasma and Serum

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Summary: Determination of homocysteine in plasma or serum for evaluation of cobalamin and folate deficiency is becoming an important diagnostic procedure. Accurate, rapid and low cost methods for measuring homocysteine are therefore required. We have improved an HPLC method and made it suitable for clinical application. The more important changes are the addition of an internal standard, mercaptopropionylglycine, and the use of a plasma/serum based calibration material. The method consists of the following steps: reduction of the sample with tri-*n*-butylphosphine, precipitation of proteins, derivatisation with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate, and HPLC separation followed by fluorescence detection. The linearity of the assays is established and the coefficient of variation is 3.0%. Stability studies show that blood samples must be cooled or centrifuged immediately after venipuncture. The method is useful for evaluation of cobalamin or folate deficiency, especially in patients with normal or moderately depressed cobalamin or folate concentrations in blood.

Introduction

Homocysteine is intimately related to methionine metabolism. It constitutes a link between the transmethylation cycle where homocysteine is remethylated to methionine and the transsulphuration pathway which catabolises homocysteine to sulphate. Cobalamin and folate defiency results in impaired homocysteine remethylation and homocysteinaemia. The increasing interest in measuring homocysteine is related to its correlation to the cobalamin and folate status in patients deficient in one of these substances, and also to its presumed role in arteriosclerotic cerebrovascular diseases. Recently, these and other subjects related to homocysteine in plasma were reviewed by *Ueland & Refsum* (1).

Our method can be used in any laboratory equipped with an HPLC system, and all reagents are commercially avalabile. Additon of mercaptopropionylglycine as an internal standard serves a double function in the assay – both as a control for the derivatisation reaction and as a standard for the size of the homocysteine peak.

About 70% of the homocysteine in plasma is reported to be bound to albumin (1, 2) and a variable amount is present as mixed disulphides. To measure the total homocysteine content (i.e., the sum of free homocysteine and disulphide-bound homocysteine) the plasma/serum sample is reduced with tri-*n*-butylphosphine before protein precipitation. The content of free homocysteine can be measured by performing the reduction step after protein precipitation.

Materials and Methods

Reagents

Ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate was obtained from Wako (Kyoto, Japan); *DL*-homocysteine, mercaptopropionylglycine and tri-*n*-butylphosphine was from Sigma (St. Louis, MO, U.S.A.); cysteine and glutathione was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Apparatus

Separation and quantification were performed with a Merck Hitachi system (Pump 655A-12, LC Controller L-5000, fluorescence detector F-1050 and autosampler 655A-40) equipped

with a Merck LiChrospher 100 RP-18 column (4 × 125 mm, 5 µm particles) protected by a Merck LiChrospher 100 RP-18 guard column (4 × 40 mm, 5 µm particles). The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. The detection signal was recorded and the peak areas quantified with a Merck Hitachi D-2000 integrator.

Chromatographic conditions

We used the gradient procedures described by *Araki & Sako* (2). Although our column and HPLC system differs from theirs and we observe shorter retention times, the procedures work satisfactorily.

Buffer A: 0.1 mol/l acetate buffer, pH 4.0, containing 20 ml/l methanol (prepared from 0.1 mol/l acetic acid and 0.1 mol/l sodium acetate, approximately 600/1500).

Buffer B: 0.1 mol/l phosphate buffer, pH 6.0, containing 50 ml/l methanol (prepared from 0.1 mol/l sodium dihydrogen phosphate and 0.1 mol/l disodium hydrogen phosphate, approximately 625/125).

The buffers were filtered through a 0.45 µm Millipore filter. The linear gradient was run from solvent A to solvent B over 12.5 min (then 2.5 min B, a 3.0 min gradient back to A, and 2.0 min A before the next injection) at a flow rate of 1.00 ml/min. We also used an isocratic system, which was run for 20 min with solvent A at a flow rate of 1.00 ml/min.

Sample preparation

Plasma samples were obtained from about 3 ml of whole blood, which were collected in a Venoject tube containing EDTA, then immediately cooled on ice and centrifuged as soon as possible at 3000 min^{-1} (2000 *g*) for 10 min at room temperature. Serum was prepared from whole blood which was left to clot for ½ – ¾ hour at room temperature, then centrifuged. Specimens were stored at 4 °C for analysis on the same day. Storage for longer periods was at −20 °C.

One hundred and fifty microlitres of plasma/serum (sample or calibration material) was mixed with 50 µl of buffer/standard (0 or 0.2 mmol/l homocysteine, 0.2 mmol/l mercaptopropionylglycine in 0.1 mol/l potassium borate, pH 9.5, containing 2 mmol/l EDTA). Twenty microlitres of 100 ml/l tri-*n*-butylphosphine in dimethylformamide were added, and the reduction allowed to proceed for 30 min at 4 °C. Samples were then mixed with 125 µl of 0.6 mol/l perchloric acid, containing 1 mmol/l EDTA, left at room temperature for 10 min, then centrifuged at 13 000 min^{-1} (15 500 *g*) for 10 min. One hundred microlitres were taken from the middle of the supernatant and mixed with 200 µl of 2 mol/l potassium borate, pH 10.5, containing 5 mmol/l EDTA. Ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate solution (100 µl) (1.0 g/l of 2 mol/l potassium borate, pH 9.5) were added and the mixture incubated at 60 °C for 60 min. After cooling in an ice bath, the samples were ready for HPLC. Ten microlitres of derivatised sample were used for each injection.

Specimens

Blood was obtained in the morning from 16 apparently healthy volunteers (eight men and eight women, aged 25–60 years). All were in a good nutritional state with a normal protein intake. None took drugs.

To test for possible interference from drugs, we measured homocysteine in plasma samples from 60 patients who had received various analgesic, antiepileptic, cardioactive, antipsychotic, antibiotic or antineoplastic drugs.

Calibration and calculations

A two point calibration line was obtained from triplicate analysis of a plasma/serum pool, and of this pool spiked with 50 µmol/l homocysteine. A linear regression line, with the added homocysteine as the abcissa and the ratio between the area of the homocysteine peak and the mercaptopropionylglycine peak as the ordinate, was calculated to determine the slope of the calibration line. The concentration of homocysteine in the samples was then determined by dividing the ratio between the area of the homocysteine peak and the mercaptopropionylglycine peak by the slope of the calibration line.

The plasma and serum pools were stored as aliquots at −20 °C. The homocysteine and the mercaptopropionylglycine solutions were kept as 0.4 mmol/l aliquots at −20 °C.

Results and Discussion

Optimizing the assay conditions

In this work we have improved a method published by *Araki & Sako* (2) and made it more suitable for routine clinical use. The most important changes are the use of a plasma/serum pool for calibration instead of the buffer, and the addition of an internal standard. The use of a plasma/serum pool is necessary because the slope in the buffer calibration is less than in the plasma/serum pool calibration. The addition of mercaptopropionylglycine as an internal standard serves two purposes, as a control of the derivatisation procedure and as a direct standardisation of the size of the homocysteine peak. If the internal standard mercaptopropionylglycine is not added to the samples we find that the total imprecision of the method approximately doubles. Other technical changes are the use of 150 µl of sample instead of the 500 µl used by the previous method (2) and precipitation with perchloric acid instead of trichloroacetic acid. In our system, the use of perchloric acid instead of trichloroacetic acid gives a better separation of the peaks. As mentioned, we also tried to run the samples with an isocratic system to check whether HPLC systems not equipped with a gradient system can be used. The chromatograms obtained in this way with buffer A only are very similar to those from a gradient run, but we have not checked the stability and the durability of the column under these conditions for a longer period.

Specificity

Derivatisation of the plasma/serum samples with the thiol-specific reagent ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (3) results in a simple chromatogram with a few well separated peaks as shown in figure 1. The retention time for homocysteine is 3.2 min (SD = 0.032 min, CV = 1.0%, n = 100) and for the internal standard 15.1 min (SD = 0.16 min, CV = 1.1%, n = 100).

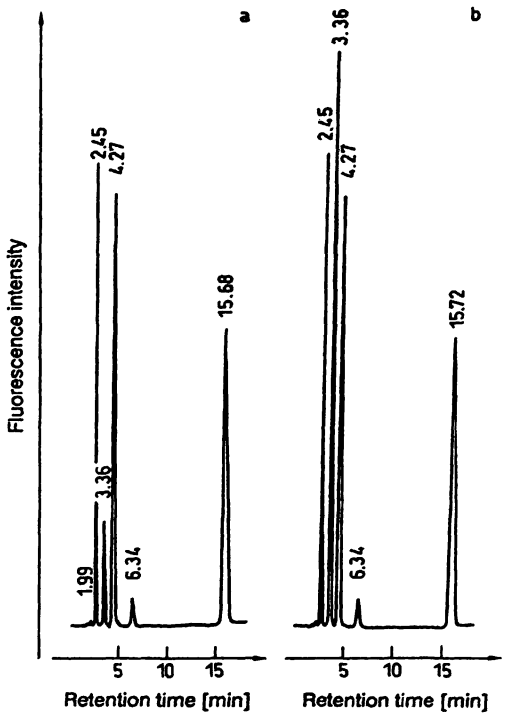


Fig. 1. HPLC chromatograms of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate derivatised plasma with added internal mercaptopropionylglycine standard.
a) Plasma pool used for calibration.
b) Plasma pool with 50 μmol/l homocysteine added.
The peaks at 3.36 min are the homocysteine and the peaks at 15.68 and 15.72 are the mercaptopropionylglycine standard. The peaks at 2.45 min and 6.34 min are cysteine and glutathione. The peaks at 4.27 min are assigned to cysteinyl-glycine (2).

The homocysteine peak was identified by adding homocysteine to the sample and observing an increase of the symmetrical well-defined peak. In the same way, we identified the peak with a retention time of about 2.4 min as cysteine, and the peak at about 6.3 min as glutathione. By comparison with the original work of *Araki & Sako* (2) we tentatively identify the peak at about 4.3 min as cysteinyl-glycine.

When we analysed plasma samples from 60 patients who had received various drugs, the chromatograms did not reveal any interferences in the assay or the appearance of new peaks.

Calibration

Calibration lines prepared by the analysis of homocysteine in 0.2 mol/l potassium borate, pH 9.5, or in a plasma pool showed different slopes (15% difference). Calibration must therefore be performed with plasma/serum-based calibrators. This matrix effect could be due to the presence of proteins, ionic strength or some other species catalysing the derivatisation step. The variation of the slope in different plasmas was also investigated; the greatest variation was 4% and thus less than the difference between a plasma pool and the buffer.

Recovery and linearity

To investigate the recovery and linearity of the assay, we added different concentrations of homocysteine to both the plasma and serum pools. Homocysteine was added to a plasma pool so as to increase its concentration by 0, 25, 50, 100, 200, 300, 400 and 500 μmol/l. Linear regression analysis of the results gave $y = 0.0220 + 0.0124\,x$ with a correlation coefficient of 0.999, where y is the ratio of the homocysteine peak area divided by the mercaptopropionylglycine peak area and x is the increase in homocysteine concentration. The same experiment with serum gave $y = 0.1015 + 0.0136\,x$ with a correlation coefficient of 1.000. Addition of 0, 5, 10, 25, 50 and 100 μmol/l to a plasma pool gave $y = 11.65 + 0.823\,x$ with a correlation coefficient of 0.999, where y is the ratio of the homocysteine peak area and x is the increase in homocysteine concentration.

Plasma and serum samples were also diluted 1.2, 1.5, 2, 3, 6 and 12 times and plotted with x as the amount of sample and y as the homocysteine peak area divided by the mercaptopropionylglycine peak area. The resulting line is slightly curved as expected from the dilution of the samples with a buffer with a 15% lower standardisation slope.

If a patient sample contains so much homocysteine that the chromatogram response goes off the scale (in our system more than 500 μmol/l), the injection sample can be diluted and chromatographed again and recalculated by using the area of the mercaptopropionylglycine peak multiplied by the dilution factor.

Detection limit

The sensitivity of the method when defined as a signal to noise ratio ≥ 5 is at least 0.3 μmol/l homocysteine. This value is in accordance with other HPLC methods for measuring homocysteine (3, 4). *Araki & Sako* (2) did not state a limit of detection for measuring total homocysteine.

Precision

The total imprecision of the method was determined by dividing a plasma sample into aliquots and determining the homocysteine concentration of each aliquot in duplicate over a period of four weeks. The coefficient of variation was 3.0% (mean = 8.92

μmol/l homocysteine, n = 11). The within-run component of imprecision was 2.3%. *Araki & Sako* (2) did not report values which are directly comparable. Furthermore, we divided a sample pool into ten aliquots which were assayed in one run. The coefficient of variation was 2.4% (mean = 10.94 μmol/l). The major part of the variability is thus in the within-run imprecision, implying a low day-to-day variation.

The within-run imprecision of our method compares favourably with those reported for other HPLC assays for homocysteine in plasma, namely 2.9% (3), 5.0% (4), and 3.8% (5). This improvement is probably a result of the addition of the internal standard. Mercaptopropionylglycine has a high chemical resemblance to homocysteine although the retention time for mercaptopropionylglycine is considerably higher than for the homocysteine peak.

Stability studies

We investigated the effect of incubation of whole blood at room temperature before separation of plasma or serum from cells. As depicted in figure 2, a fresh blood sample left at room temperature without centrifugation will show a considerable increase in homocysteine concentration. This increase is linear for at least 4 hours and amounts to 9–10% per hour. The relative increase after 24 hours varies significantly from sample to sample. This increase in homocysteine concentration can be eliminated by centrifugation followed by pipetting of plasma/serum. After centrifugation and pipetting the concentrations of homocysteine in the samples are stable, even at room temperature, for at least 24 hours. Therefore, the blood samples must be cooled or centrifuged immediately after blood sampling.

We stored plasma/serum samples at −20 °C for three months without any measurable change in the homocysteine concentrations. Furthermore, one sample was thawed and frozen nine times during one week and each time an aliquot was removed and frozen. When all samples were analysed in the same run, the homocysteine concentration was 9.64 ± 0.19 μmol/l (mean ± SD) and the first and last values were 9.67 and 9.51, respectively.

The samples are also very stable after derivatisation with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate. They can be stored at 4 °C for several weeks. The samples may turn yellow because ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate is light sensitive. However, this does not seem to cause any problems in the assay, but as a precaution we kept the ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate solution in a cold and dark place until use.

We did not filter the samples before injection into the column because we found it more economical (both in materials — the cost of one guard column equals the cost of 20 filters — and working hours) to change the small guard columns after about 75 sample injections.

The high increase of homocysteine in plasma/serum when blood samples are left at room temperature is an interesting problem. Cooling of the blood sample followed by incubation at room temperatures does not stop the increase of homocysteine in plasma/serum. We measured the homocysteine concentration in haemolysates of blood and in isotonic NaCl incubated with the residue of blood samples after removal of plasma. These preliminary experiments indicate that the homocysteine increase stems from a produc-

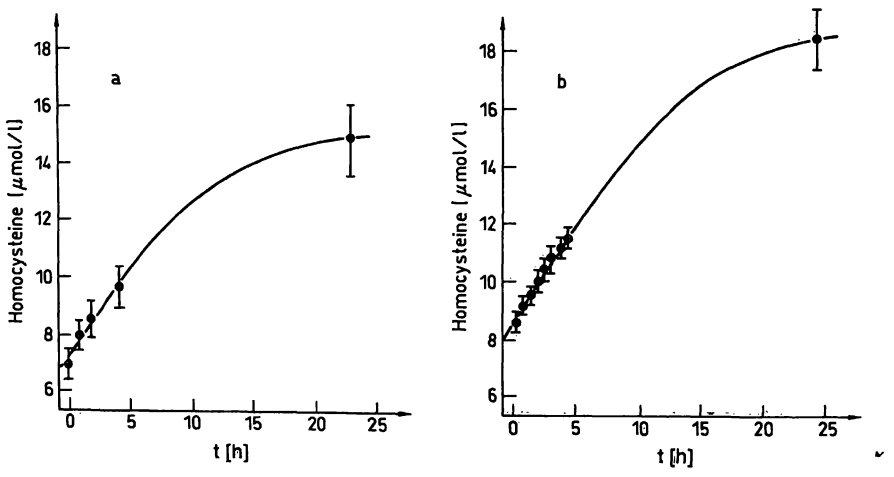
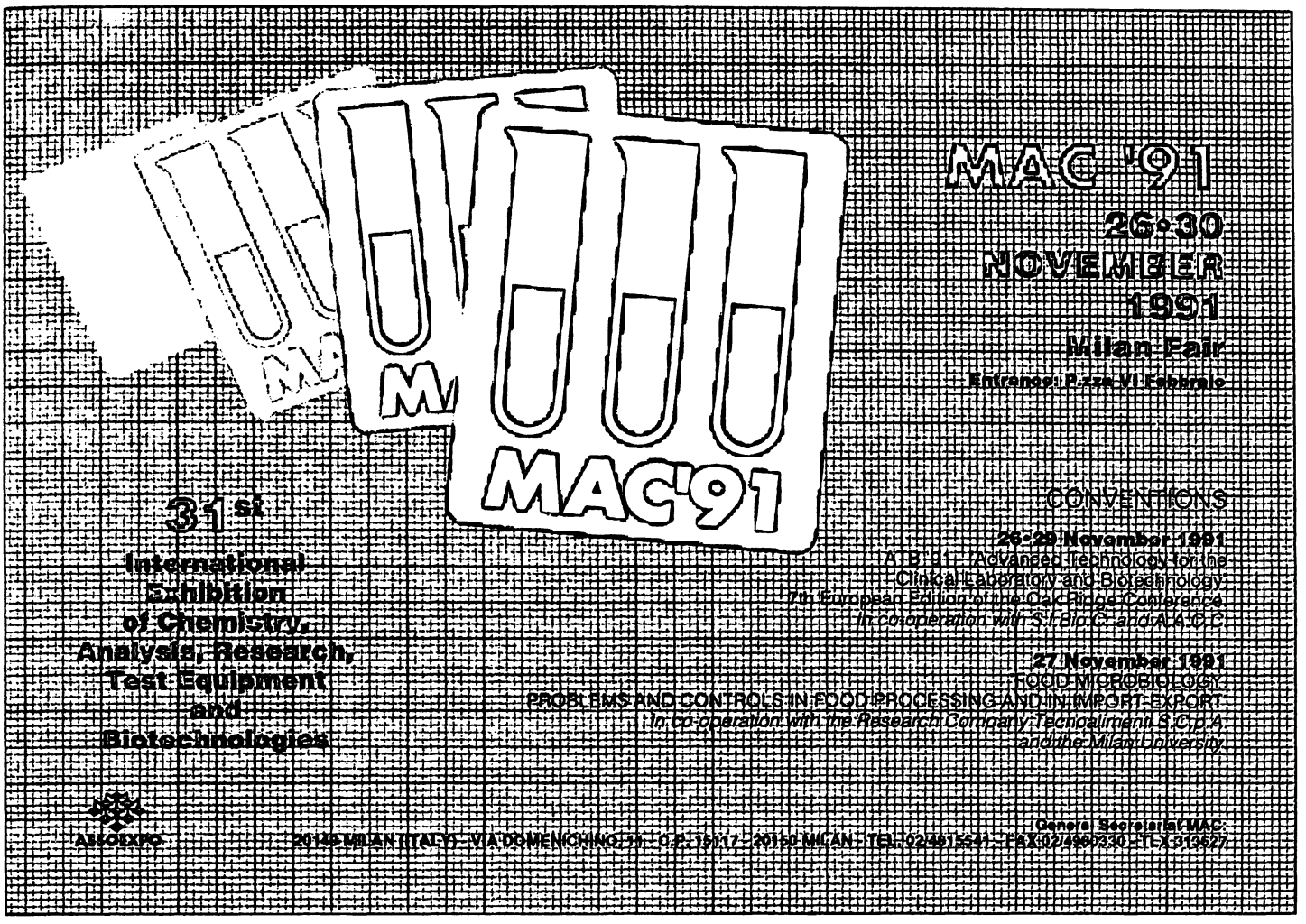


Fig. 2. Incubation of whole blood at room temperature before separation of plasma or serum from cells. The increases of homocysteine when measured in plasma (a) or serum (b) specimens, are depicted. The results are the average of experiments on five subjects. The error bars indicate ± SEM.



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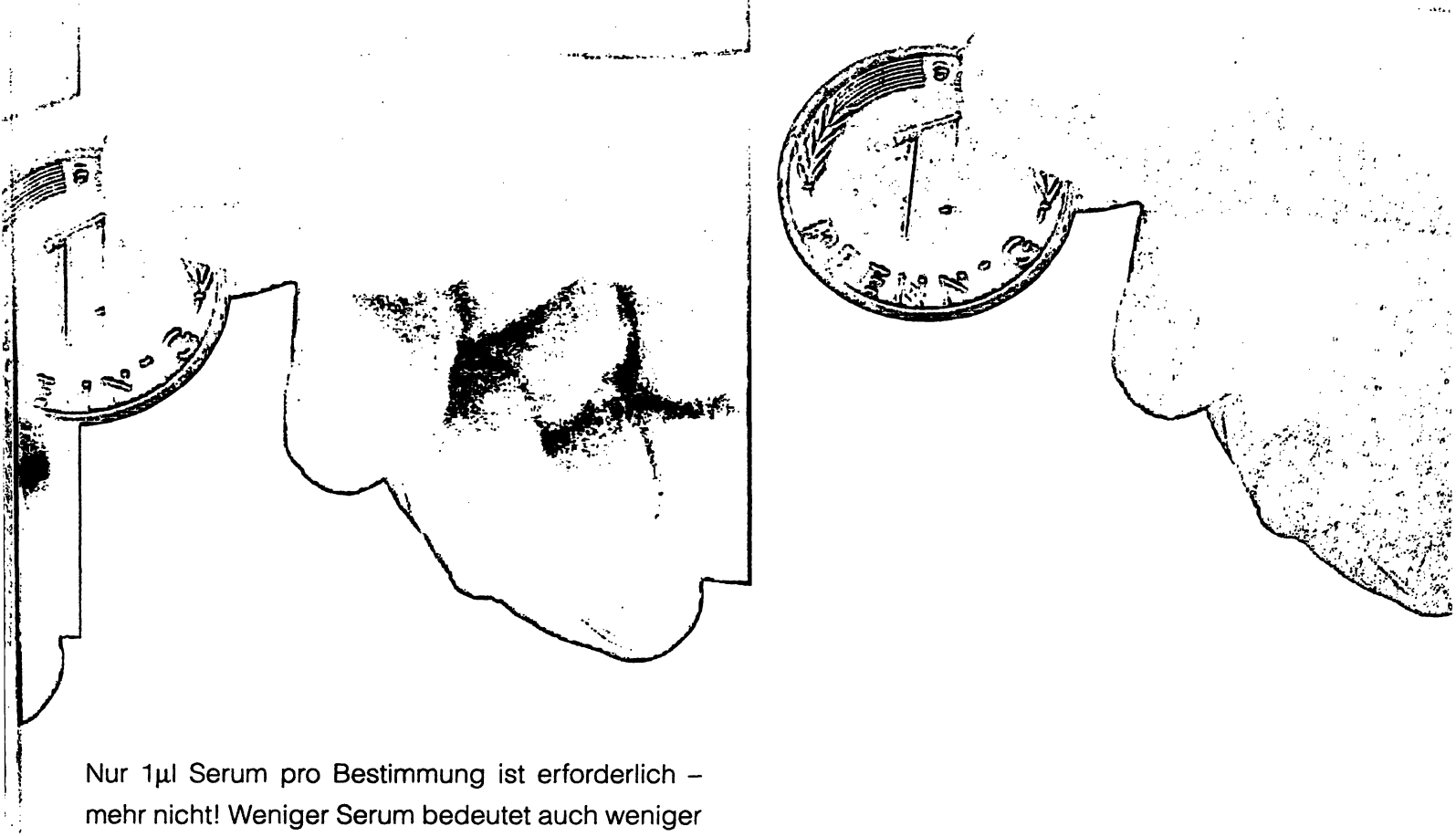

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Editors M. Y. Kalimi, W. Regelson

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tion of homocysteine or a conversion of homocysteine derivatives to homocysteine in the blood cells. This matter will be further investigated.

Practicability

Whether it is preferable to use plasma or serum is very dependent on the laboratory routines. We prefer to use plasma, because although serum can be used if the reference range for healthy individuals is adjusted to it (tab. 1), it is impractical to keep control of the clotting time and the following centrifugation. The plasma method requires "a glass of ice at the bedside" and although this is inconvenient it is manageable.

Tab. 1. Homocysteine concentration in μmol/l, analysed in plasma and serum obtained from the same venipuncture. n = 8 for both men and women.

	mean	SD	range
Plasma			
men	9.8	1.6	7.8–11.9
women	6.8	1.2	5.5– 8.4
Serum			
men	10.8	1.6	8.7–13.0
women	7.6	1.2	5.8– 9.1

About 50 tubes (calibrators + 22 samples in duplicate) can easily be processed in one working day and the samples can be analysed overnight. Use of the assay for six months provided it to be a robust and effective method, suitable for routine analysis.

We also used the method to determine free homocysteine in two samples (by precipitating the proteins before reduction with tri-*n*-butylphosphine). The samples showed a free homocysteine content of 1.6 μmol/l and 1.7 μmol/l homocysteine (6.1 μmol/l and 6.9 μmol/l in total homocysteine) which compare well with the values reviewed by *Ueland & Refsum* (1).

Analysed blood samples

Plasma/serum homocysteine concentrations were measured in blood samples from 16 apparently healthy volunteers. The plasma and the serum were obtained from the same venipuncture, and the homocysteine values were consistently higher (in average 10%) for all serum samples ($p < 0.005$, *Wilcoxon* test for pair differences). Most of this difference can be attributed to the different treatment of the blood samples, plasma samples being cooled immediately, whereas serum samples are left to clot at room temperature for ½ – ¾ h before centrifugation.

The homocysteine range has been reported to differ between men and women (as illustrated by the values below) but also to be age-dependent; for further details see l. c. (1). In general, men seem to have higher concentrations of homocysteine than women, while older people have higher concentrations than younger people. Our data, which are summarized in table 1, support this difference between the homocysteine values in men and women ($p < 0.005$, *Wilcoxon* test for two samples). Our values are a little higher than those reported by *Araki & Sako* (2) who found 6.53 ± 1.08 μmol/l homocysteine (mean ± SD) for men (n = 20), and 5.71 ± 1.20 for women (n = 15), while they are a little lower than the values reported by *Brattström* et al. (6) who reported 12.1 ± 4.0 for men (n = 22) and 8.9 ± 1.0 for women (n = 10). Even higher values (7.2–21.7 μmol/l homocysteine (mean ± 2 SD), 20% higher in males than in females) have been reported for serum samples (7). These differences can probably be explained by the different handling of the blood samples before separation of plasma and serum from cells and the various analytical methods employed.

Finally, in plasma samples of two patients with depressed concentrations of erythrocyte folate (188 and 301 nmol/l respectively; reference range: 400–1200 nmol/l) we found increased concentrations of plasma homocysteine, namely, 60.6 and 48.1 μmol/l.

Conclusion

We have improved a method for measuring total homocysteine in plasma and serum. The method uses only commercially available reagents and requires only a standard HPLC apparatus equipped with a fluorescence detector in addition to normal laboratory equipment. The addition of an internal standard ensures good precision, accuracy and performance. The analysis is robust, fast and inexpensive and is now suitable for routine use in the clinical laboratory.

Acknowledgement

We thank Dr. *E. Mortensen* for helpful discussions, Dr. *H. Jelert* for his assistance in obtaining the blood samples from patients together with information about disease and drug intake, and *A. L. Christensen* for skilful technical assistance.

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